Promoter methylation in the *PTCH* gene in cervical epithelial cancer and ovarian cancer tissue as studied by eight novel Pyrosequencing® assays

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Abstract. DNA methylation status in the CpG sites of promoter regions in cancer-related genes, such as *PTCH*, has traditionally been investigated using either dye-terminator sequencing or methylation-specific PCR. We aimed to study the *PTCH* gene promoter methylation in gynecological cancers, with a method that gives a quantitative measure of the methylation status of the promoter region of the studied gene, and for this purpose, we designed novel Pyrosequencing-based assays. Bisulfite-treated genomic DNA (bsDNA) was amplified by standard PCR and applied to novel Pyrosequencing® assays, in order to measure the methylated fraction (%) at each CpG site of the *PTCH* gene promoter. We analyzed 22 squamous cell cervical cancer tissue specimens (11 with good and 11 with poor outcomes after radiotherapy) and 5 ovarian cancer tissue specimens matched with 5 normal ovarian tissue specimens. Six optimized PCR protocols which generated 8 Pyrosequencing assays covering 63 CpG sites in the promoter regions 1 and 2 as well as the previously unanalyzed promoter region 3 in the *PTCH* gene were developed. The 27 tumor tissue specimens and 5 normal tissues did not show any methylation within any of the 63 CpG sites. Our data suggest that methylation of the *PTCH* promoter is not a high-prevalence feature of squamous cell cervical cancer or ovarian cancer, but Pyrosequencing assays are a good method for studying promoter methylation.

Introduction

The Patched gene (*PTCH*) is involved in the Hedgehog/Patched signaling pathway and is known to play a role in mammalian development and in regulation of stem cell renewal in adult tissue (1). *PTCH* is a major pathway receptor localized to the plasma membrane that moves to the endocytic vesicles upon ligand binding (2,3), where the ligand could be either Sonic (SHH), Indian (IHH) or Desert Hedgehog (DHH). PTCH associates with the Smoothened protein (SMO) and inhibits downstream targets such as *PTCH, GLI1, HIP* and *TGF-β* (4) in the absence of ligand. PTCH activity is repressed and SMO is activated if SHH is present. This activity leads to the translocation of the GLI protein to the nucleus and transcription of downstream targets (5).

Alterations in this pathway such as phenotypical changes of the proteins involved because of mutations of the genes (2,6), or dysregulation of the genes because of aberrant methylation patterns of their promoter regions (7,8), can alter carcinogenic transformation.

PTCH is expressed in the adult human kidney, liver, lung, brain, heart, placenta, skeletal muscle and pancreas (9). It has been suggested that the *PTCH* gene has tumor-suppressor function because it appears to be involved in the development of several cancers such as ovarian dermoids, fibromas (7), medulloblastoma (10), acute myeloid leukemia (11), uterine cervical carcinoma (12), breast cancer (8), sporadic basal cell carcinomas (13) and nevoid basal cell carcinoma (2).

Epigenetic regulation, through methylation or demethylation of CpG sites in promoter regions of cancer-related genes by methyltransferases (14), plays an important role in the pathogenesis of cancer (15). This mode of regulation can be considered activating or silencing depending upon action. *PTCH* has several alternative splicing sites (a, b, c, d and e) starting from exon 1 (16,17). The most studied of these regions, in relevance to epigenetic changes and regulatory effects of the protein (7,8,12), contains the N-terminus encoded by exon 1B (16,18). This particular variant is known to have full inhibitory activity on SMO (18). Recent data suggest an enhanced expression of the PTCH protein in squamous cell carcinoma of uterine cervix and its precursor lesions (12). To link this finding to possible changes in methylation status of the promoter regions of *PTCH*, in response to ovarian cancer development, is an overarching goal of our current *PTCH* studies using dye-terminator sequencing. Recent findings provide evidence of hypermethylation within the 1600-bp

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long *PTCH* gene promoter (7), which was divided into four large overlapping contigs. Results were generated from regions 1, 2 and 4; however, region 3 was found to be unfeasible to amplify (7). Dye-terminator sequencing generates a qualitative assay output (presence or absence of methylation); therefore, it is considered limited. This methodology is also generally considered more labor intensive and more costly than other DNA methylation analysis techniques.

Our goal in the present study was to dissect the promoter region of the PTCH gene into a larger number of short amplicons, in order to investigate the methylation status of the CpG sites in region 3 that have not yet been studied. In addition, we aimed to quantitatively determine the methylated fraction of the remainder of the CpG sites in the promoter region of the *PTCH* gene (Fig. 1). Therefore, we designed new bisulfite-specific PCR (bs PCR) primers and new Pyrosequencing assays to quantify the methylated fraction of 63 CpG sites contained within the promoter region of the *PTCH* gene. The assays were applied to a clinical sample of cervical cancer tissues, selected from patients with poor or good prognosis respectively, and to a sample of ovarian cancer tissues with matching normal ovarian tissue controls.

Materials and methods

Subjects. EDTA blood plasma samples from our routine laboratory were de-identified and used to optimize methods to study the methylation status of selected CpG sites in exon 1b in the promoter region of the *PTCH* gene. According to Swedish Research Ethics Law on anonymized samples for method development purposes, informed consent was waived for these samples. The novel methods were subsequently used in two clinical studies: a) 22 squamous cell cervical cancer tissue specimens obtained before start of primary radiotherapy of which 11 were from women with a poor prognosis (recurrence of cervical cancer after radiotherapy) and the other 11 from women with a good prognosis (no recurrence of cervical cancer after radiotherapy). The two groups were 100% matched for tumor stage. The Regional Ethics Committee in Uppsala approved the Swedish part of the study (Act No. 2008/089, 25th june 2008, Uppsala, Sweden); b) 5 de-identified ovarian tumour tissue specimens and 5 normal ovarian tissue specimens from the same patients (kindly provided by Dr S. Oreskovic, Zagreb University Hospital).

The Ethics Committee of the University Hospital Centre Zagreb, Department of Gynaecology and Obstetrics and School of Medicine University of Zagreb on March 1st, 2006 gave statement on approved protocols for genetic research on clinical samples from Department of Gynecology and Obstetrics (Act No. 021-1/49-2006, Zagreb, Petrova 13).

DNA isolation and bisulfite treatment. Genomic DNA (gDNA) was extracted in three different ways. Either from 200 μl whole EDTA blood using a BioRobot EZ1 (Qiagen Inc., Valencia, CA, USA) with the QIAamp EZ1 DNA blood 200 μl kit according to the manufacturer's instruction (Qiagen), otherwise from squamous cell cervical cancer tissue pieces using the QIAamp DNA Mini Kit (Qiagen) or from ovarian cancer tissue pieces using standard phenol-chloroform method. In the latter method, all samples were first digested in buffer (0.9% Tween-20, 0.9% Triton X-100, 5 mmol/l EDTA, 2 mmol/l DTT, 10 mmol/l TrisHCl pH 7.5) and proteinase K (20 μg/ml) overnight at 55˚C.

Approximately 1000 ng freshly extracted DNA was used for the bisulfite treatment. The bisulfite treatment was performed with the EZ DNA Methylation kit according to the manufacturer's instructions (Zymo Research, Orange, CA, USA); however, the incubation time was shortened to 10 h if the DNA originated from tissue and not from blood. In summary, DNA was diluted with M-Dilution buffer and incubated for 15 min at 37˚C. CT conversion reagent was added to the DNA samples from the previous step and incubated again at 50˚C for 16 h (10 h if the DNA originated from tissue). Finally, the samples were incubated on ice for 10 min and then M-Binding buffer was added. The samples were centrifuged and then washed with M-Wash buffer. The bsDNA was eluted in 10 μl M-Elution buffer and then diluted 5 times with TE buffer (10 mmol/l Tris-HCl, 0.05 mmol/l EDTA, pH 7.5).

bs PCR. PCR primers for Pyrosequencing were designed using Methprimer (http://www.urogene.org/methprimer/index1.html). Primer sequences can be found in Table I. The objective of the PTCH-gene-PCR primer design was to amplify the same area and same CpG sites as previously studied by Cretnik *et al* (7). We chose to shorten the amplicons to be able to perform Pyrosequencing analysis of the CpG sites, a technique where ~150-200 bp is the optimal amplicon length (19). The PCR primers would also be uniquely designed and devoid of CpG sites in primer-binding areas such that amplification will always take place independent of methylation status. This is also known as bisulfite-specific PCR (bs PCR). We utilized the same numbering scheme of CpG sites as reported by Cretnik *et al* (7) and as shown in Table I and Fig. 1.

PCR was performed using an Eppendorf Mastercycler (Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany) and the HotStarTaq DNA polymerase Kit (Qiagen); we used 30 or 60 μl volume reactions depending on the number of amplicons required to analyze in the Pyrosequencer (PSQ 96MA system, Biotage AB). Each reaction contained 0.4 μmol/l of each primer, 1.25 U of Taq polymerase, 1.5 mmol/l $MgCl_2$ and 0.2 mmol/l each of dGTP, dATP, dTTP and dCTP. Five μl of the diluted bisulfite-treated gDNA served as the PCR template. The PCR program consisted of an initial polymerase activation step at 95˚C for 15 min followed by 53 cycles of denaturation at 94˚C for 30 sec, primer annealing at 48-57˚C for 30 sec and extension at 72˚C for 1 min. A final extension step at 72˚C for 7 min finalized the program. The PCR primers, annealing temperatures and amplicon sizes for Pyrosequencing are shown in Table I.

Pyrosequencing. To quantify site-specific methylation, PCR products from the bisulfite-treated genomic DNA samples were analyzed with Pyrosequencing technology; the sequence primers are summarized in Table I. The post-PCR (see above) samples were prepared with the Vacuum Prep Workstation (Biotage AB, Uppsala, Sweden) according to the following protocol summary: 30 μl of the amplicon, 3 μl Streptavidin

Table I. PCR primer sequences, annealing temperatures, $MgCl_2$ concentration, amplicon sizes and sequencing primer sequences for 6 different amplicons of the *PTCH* gene.[a]

Amplicon & CpG site no.	PCR primer sequence (5'-3')	Annealing temperature (˚C)	$MgCl_2$ concentration (mmol/l)	Size (bp)	Sequencing primer (5'-3')
Amp2	F: Biotin-TTATAAAAAGAAGATATTGTTGAAAAGAAA	53	2	163	Py1: AACAACCCAATATATACATCCT
Site 13-16	R: AAATAAAAAAACCACCCAAATAAAC				
Amp3	F: TAGTTAATAATAGTTATGTTTGTTGAGTAA	48	2	187	Py1: GTTTGTTGAGTAATTTTTGTTT
Site 17-23	R: Biotin-AAAAACTCTCTCCATTTAAAAAAAA				
Amp4	F: TTTTTTTTAAATGGAGAGAGTTTTT	55	2	188	Py1: TATTGAATTAAGGAGTTGTTG
Site 26-36	R: Biotin-AACTCTACTTTCTTATACTCCT				Py2: GGAGTTTTTAGGTTT
Amp5	F: GGGGATAGAATGGTTTAG	53	1.5	117	Py1: AGGAGTATAAGAAAGTAGAGTT
Site 37-42	R: Biotin-ACTCCAAAAACTACTACT				
Amp6	F: AGTAGTAGTTTTTGGAGT	50	1.5	274	Py1: GTTAGTAGTAGTTAT
Site 45-60	R: Biotin-ATCCCCAACTCCCCCTACC				
Amp7	F: TTGGTTTTTTTTGTAGTGAAGGGGT	57	1. 5	198	Py1: GGTAGGGGGAGTTGGGGAT
Site 74-98	R: Biotin-CCTTCCATTACCACATTAC				Py2: GAATTGGATGTGGGTAG

[a]The amplicons are designed to cover the same area and CpG sites as previously studied by Cretnik *et al* (7). The CpG sites studied in this report are also numbered according to the previous report.

Sepharose HP Beads (Amersham Biosciences, Uppsala, Sweden), 37 μl binding buffer (10 mmol/l Tris-HCl, 2 M NaCl, 1 mmol/l EDTA, 0.1% Tween-20, Milli-Q (18.2 MΩ x cm) water, pH 7.6) and 15 μl Milli-Q water were mixed and used in the Vacuum Prep workstation. The biotinylated amplicons were immobilized onto the Streptavidin sepharose beads and then passed through one denaturation step and two washing steps using the Vacuum Prep Workstation. The first step was 70% ethanol, the second 0.2 mol/l NaOH (denaturation step) and the third was washing buffer (10 mmol/l Tris-Acetate, Milli-Q water). The amplicons were transferred to a plate containing sequencing primer (0.4 μmol/l) in 40 μl annealing buffer (20 mmol/l Tris-acetate, 2 mmol/l magnesium acetate, pH 7.6). The sequencing primer was annealed to the template at 80˚C for 2 min. Pyrosequencing was performed using the PSQ96 SNP Reagent Kit and the PSQ 96MA system (Biotage AB). Pyrograms showing the nucleotide addition order can be seen in Figs. 2-7. Pyro Q-CpG™ Software v. 1.0.9 was used to determine the optimal order of nucleotide addition, when designing the assays. The software also automatically analyzed the methylation results. The % methylated fraction (C/T ratio) is displayed in a small colored box just above each CpG site in the analyzed sequence (Figs. 2-7). Each site is analyzed as a C/T-polymorphism where a 100% C-reading denotes a fully methylated C in the original gDNA sample and a 100% T-reading denotes that this C was unmethylated in the gDNA. Intermediate C/T percentages denote partial methylation at the level of the sample.

Results

Our goal was to develop quantitative methods to measure the methylated fraction of the CpG sites in the promoter region of the *PTCH* gene without resorting to 'methylation specific' PCR. In addition, we specifically wanted to develop methods to investigate the unstudied region three of the *PTCH* promoter [Fig. 1 by Cretnik *et al* (7)]. We designed six new, shorter, amplicons that were all successfully amplified using conventional PCR with bisulfite treated gDNA as template (Table I).

We also created eight Pyrosequencing assays (Table II) that quantitatively analyzed 63 CpG sites (Figs. 2-7) in regions 1 (Fig. 1, lines 1-6 in ref. 7) and 2 (Fig. 1, lines 6-10 in ref. 7), which have been previously studied by another technique (7), and in addition we covered region 3 (Fig. 1, lines 9-15 in ref. 7) that had been previously unfeasible to study using dye-terminator sequencing (7). Table II displays the length of the PCR amplicons, how many CpG sites they cover and which of these are analyzed in our Pyrosequencing assays. Amplicons 4 and 7 are analyzed using two different Pyrosequencing assays each, whereas the others are covered using one assay each. The amplicon 7 assay includes 25 CpG sites, of which 19 are unique, while six of the analyzed CpG sites overlap with the last six CpG sites covered by the amplicon 6 assay, providing a useful internal check of the validity of results. In 96 steps, the Pyrosequencing assay, Amp6-Py1, analyses 16 CpG sites, which is rather unique, not only for this method, but also for methylation analysis in general (Fig. 6).

All the eight assays were applied to two clinical study groups comprising a total of 32 tissue samples. One sample

Figure 1. DNA sequence of the analyzed region of the *PTCH* promoter. The sequence here is shown after bisulfite conversion in which hypothetically all non-CpG sites cytosines are replaced with thymines, whereas cytosines within the CpG sites remain as cytosines. The forward and reverse primers (marked by arrows) indicate the analyzed regions. The shaded boxes mark all the 164 CpG sites counted from the first (no. 1) in the figure to the last (no. 164) spread throughout the ~1600-bp long promoter. Gli-binding sites are marked with circles and ATG is in a square.

Table II. Amplicon sizes, number of CpG sites in the templates, designation of Pyrosequencing assays and number of CpG sites analyzed for 6 different amplicons of the *PTCH* gene.

PCR amplicon	Amplicon length (bp)	No. of CpG sites in the template	Designation of the Pyrosequencing assays	No. of CpG sites analyzed by the assay
Amp2	163	4	Amp2-Py1	4
Amp3	187	9	Amp3-Py1	7
Amp4	188	11	Amp4-Py1	4
			Amp4-Py2	7
Amp5	117	8	Amp5-Py1	6
Amp6	274	38	Amp6-Py1	16
Amp7	198	25	Amp7-Py1	13
			Amp7-Py2	6

consisted of 22 squamous cell cervical cancer tissue specimens, from two groups of women: 11 with a poor prognosis (had recurrence of cervical cancer after primary radiotherapy) and 11 from women with a good prognosis (no recurrence of cervical cancer after radiotherapy). The other comprised five ovarian cancer tissues and five normal ovarian tissue

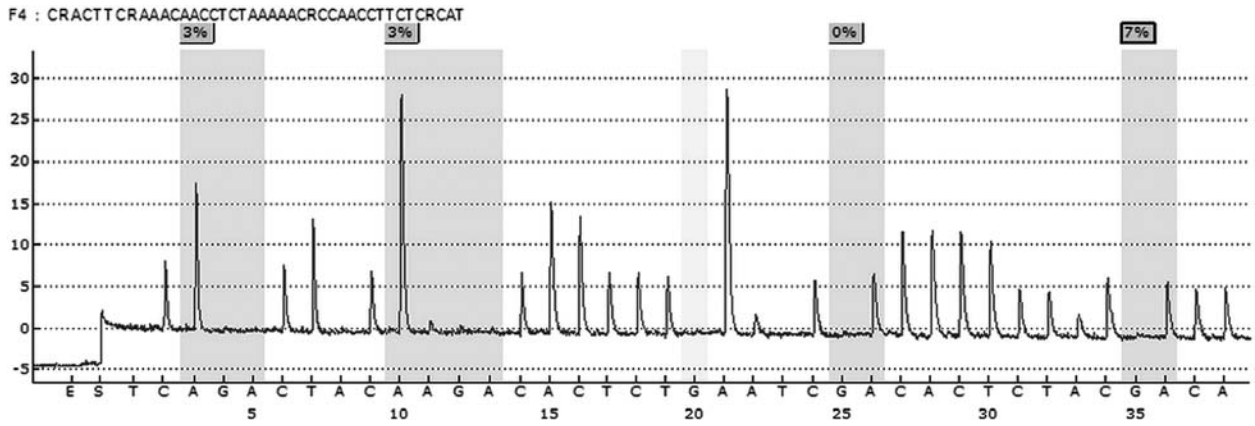

Figure 2. Typical pyrogram showing the sequence analyzed in Amp 2 in the *PTCH* gene by the Amp 2-Py1 assay. The G base added at nt 20 (lighter grey background) is a negative control to ascertain good bisulfite conversion of the sample.

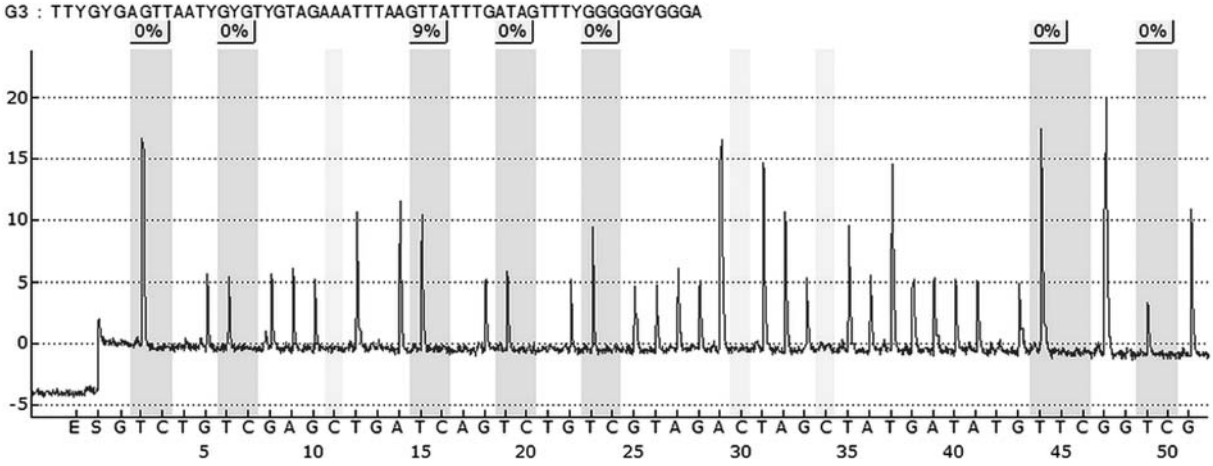

Figure 3. Typical pyrogram showing the sequence analyzed in Amp 3 in the *PTCH* gene by the Amp 3-Py1 assay.

samples, matched from each patient. None of these 32 tissue samples showed any signs of methylation in any of the eight Pyrosequencing assays.

Discussion

We optimized our PCR and Pyrosequencing protocols (Table I and Figs. 2-7) with gDNA originating from human leukocytes. This is a cell type that turned out to be unmethylated, as expected, on all CpG sites studied in the *PTCH* promoter (Figs. 2-7). Unexpectedly, none of the 22 squamous cell cervical cancer or 5 ovarian cancer tissues tested, showed methylated CpG sites in any of the eight *PTCH* assays. Therefore, *PTCH* promoter methylation is probably not a high-prevalence event in squamous cell cervical cancer or ovarian cancer. This is not a surprising finding, since it has been shown that cervical cancer samples usually show increased expression of PTCH protein (12), and the same is true for ovarian cancer (20), although LOH of the *PTCH* region has been associated with some types of ovarian cancer (21). Extended applications of these newly-developed assays to larger series of cancer tissues from other cell types will be needed to clarify the extent and role of PTCH promoter methylation in other human cancers.

The current and perhaps most popular methodology to study DNA methylation, 'methylation specific' PCR (often featured in microarrays), has the limitation of only assaying the methylation status of a few CpG sites that interfere with PCR-primer binding. This technique only gives a qualitative indication if the sites are methylated or not; like all 'allele specific' PCR methods, the judgment of the presence or absence of a band on an agarose gel is largely a matter of the combination of the number of PCR optimization experiments made and subjective analysis of gel electrophoresis bands. We believe that these drawbacks are a real cause of concern, which may have stalled progress in the understanding of the role of DNA methylation in clinical cancer research. Compared to 'methylation specific' PCR, Pyrosequencing technology and Pyro Q-CpG Software v. 1.0.9 automatically generate a quantitative measure in percent methylation for each CpG site in the studied sequence. This methodology allows the detection of partially methylated CpG sites; therefore, yielding a more accurate picture of how the methylation is distributed throughout the promoter region than the qualitative assays.

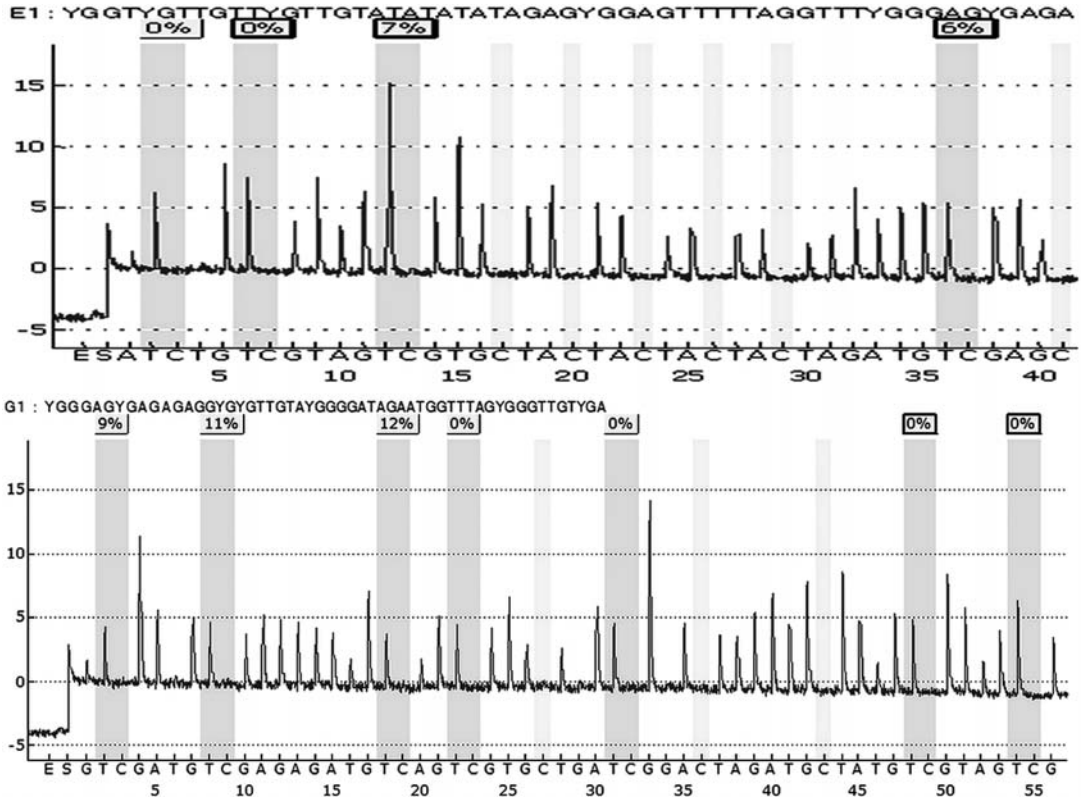

Figure 4. Typical pyrograms showing the sequences analyzed in Amp 4 in the *PTCH* gene. This amplicon was divided into two different Pyrosequencing assays, Amp4-Py1 (upper panel) and Amp4-Py2 (lower panel).

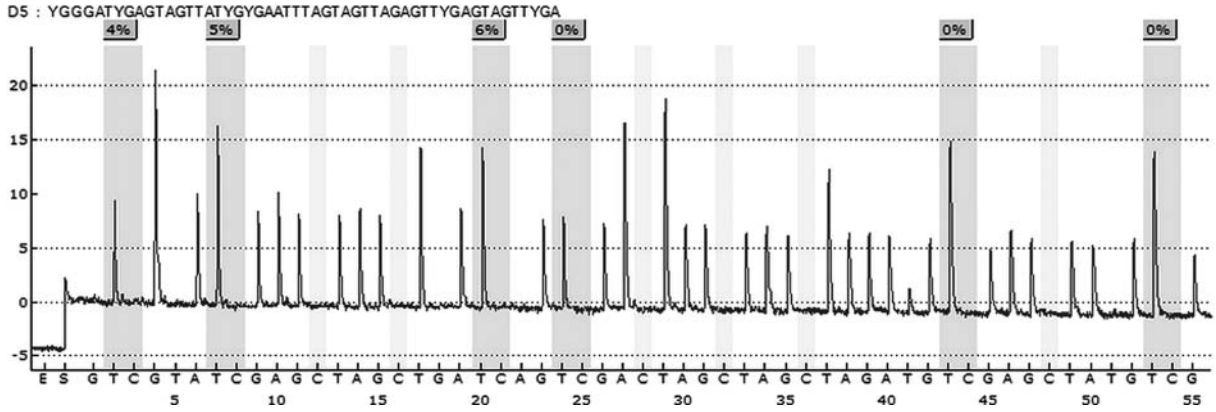

Figure 5. Typical pyrogram showing the sequence analyzed in Amp 5 in the *PTCH* gene by the Amp 5-Py1 assay.

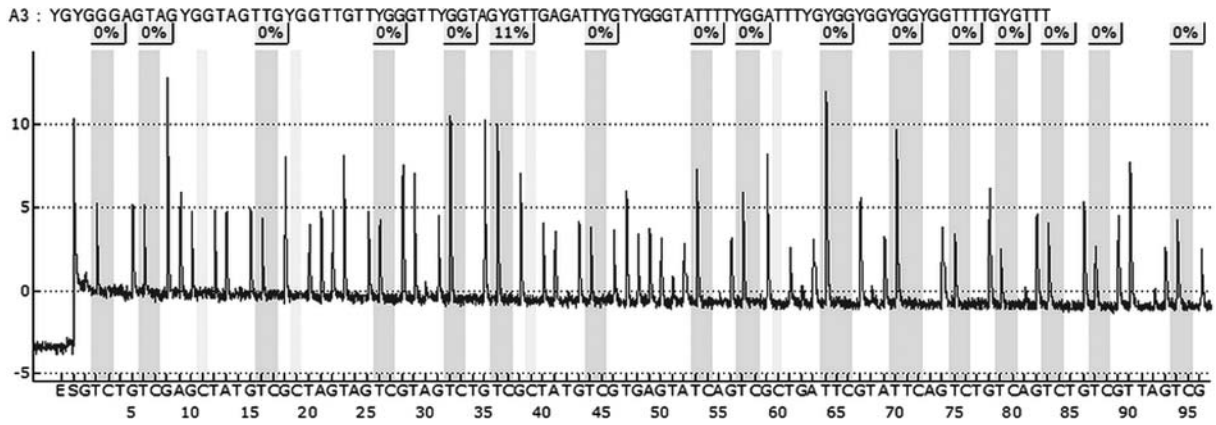

Figure 6. Typical pyrogram showing the sequence analyzed in Amp 6 in the *PTCH* gene by the Amp 6-Py1 assay.

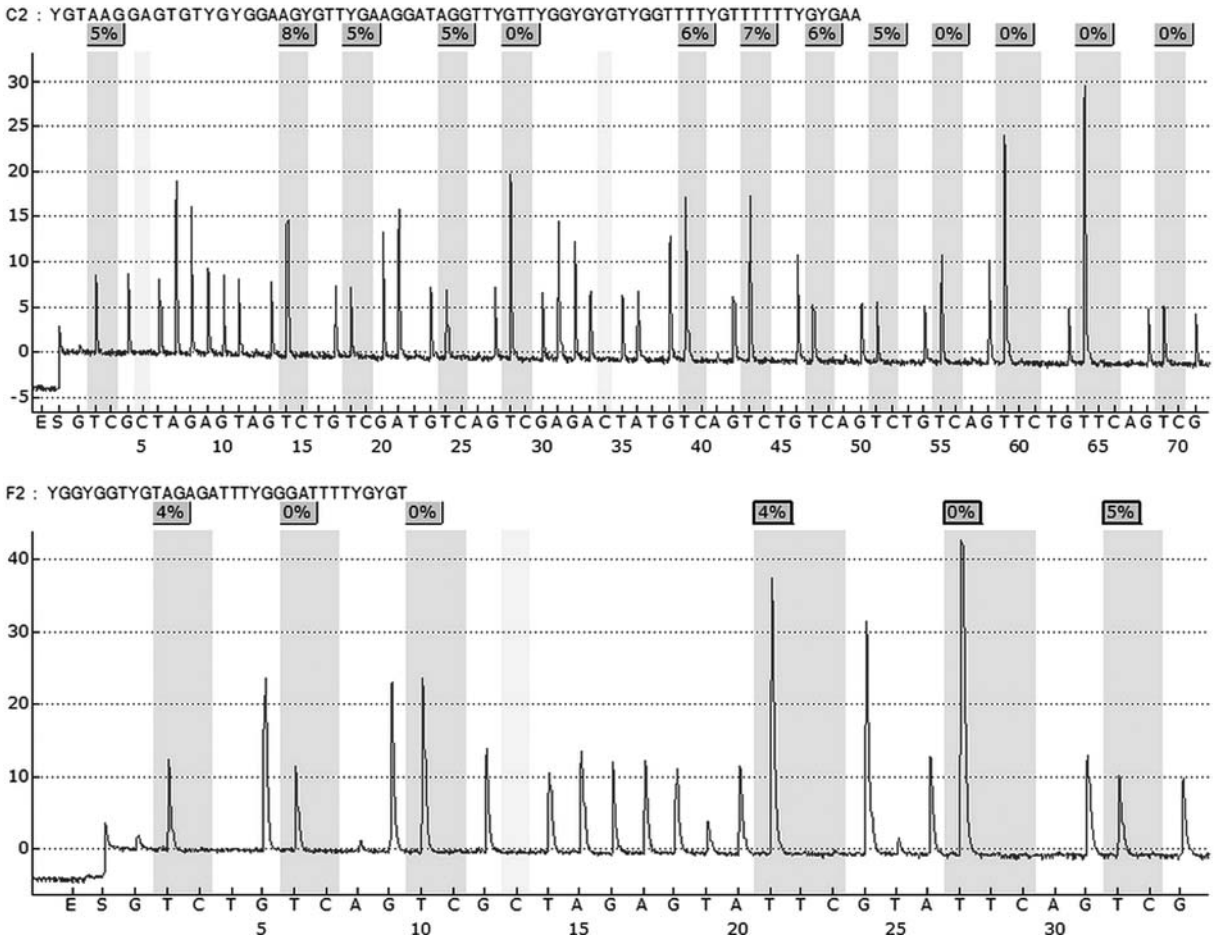

Figure 7. Typical pyrograms showing the sequences analyzed in Amp 7 of the *PTCH* gene. This amplicon was divided into two different Pyrosequencing assays, Amp7-Py1 (upper panel) and Amp7-Py2 (lower panel).

Analysis of CpG sites in the *PTCH* gene is unusually challenging owing to method development aspects, but only 36 CpG sites in the *PTCH* promoter remained uncovered by our assays designed for regions 1 and 2, recently studied by conventional DNA sequencing (7) and region 3, previously considered unfeasible to amplify. Pyrosequencing assays could not be developed for region 4 due to the extensive amount of CpG sites, including 63 sites densely grouped within a 456-bp region (Fig. 1, lines 15-20 in ref. 7): there were no stretches of unique, CpG-free sequences where PCR primers could bind; therefore, it could not be split into smaller amplicons. We also attempted to analyze this 456 bp amplicon directly by Pyrosequencing, but as expected, it turned out to be too long to analyze; optimal amplicon length for this technique is around 150-200 bp. Therefore, CpGs in that region still await to be quantified by other means.

In conclusion, we developed eight Pyrosequencing assays to quantify the methylation of promoter regions 1, 2 and the previously unanalyzed region 3 of the *PTCH* gene. Our data suggest that methylation of the *PTCH* promoter is not a high-prevalence feature of squamous cell cervical cancer or ovarian cancer. Clinical applications of the novel DNA methylation assays, in order to characterize the conditions associated with PTCH promoter methylation in various cancer forms, will be the object of our further studies.

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